Journal of Natural Sciences Research
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.4, No.2, 2014
www.iiste.org

Reduction of Some Enzymes Produced by Irradiated Fungal Strains Isolated from Certain Medicinal Plants

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Abstract
Medicinal plants normally carry high bioburden due to their origin, offering potentials hazards to the consumer. Fungal extracellular enzymes play a role in biodeterioration of medicinal plants and undesirably effect human health cause immunotoxigenic diseases. Ten different medicinal plants were screened for their mold contamination. The isolates were identified as genera *Aspergillus*, *Alternaria, Cladosporium* and *Penicillum,* they tested for their enzymatic activities (protease, cellulase and lipase). All isolates were able to produce enzymes under study in a varying degree. Of the fungal isolates , *Asp. niger* and *Asp. flavus* showed high protease activity. Whereas *P. roquefortii* and *Asp. parasiticus* were the more potent strains producing cellulase. Lipase was found to be highly produced by *Asp. fumigatus* and *P. italicum* . The present study presumes to monitor the fungal growth and enzymatic activity in relation to gamma irradiation. The results showed that, the log number of survivors was found to be inversely proportional to the irradiation dose. 6.0 and 4.0 kGy resulted in complete inhibition the growth of highly protease produces (*Asp. niger* and *Asp.flavus*). While, *P. roquefortii* and *Asp. parasiticus* which highly produce cellulase were inhibited at 4.0 and 6.0 kGy, respectively. On the other hand, *Asp.fumigatus* and *P. italicum* which highly produce lipase were inhibited at dose 4.0 and 6.0 kGy, respectively. Subleathal doses of gamma- irradiation resulted in high significant reduction of enzymes production. The stability of acquired character for the strains under study which were exposed to gamma-irradiation was studied. Statistical analysis revealed that, the enzyme activities estimated after 6 months of storage gave difference data between the strains under study. This study indicates that gamma irradiation is an effective treatment for reduction of fungi contaminating medicinal plants as well as its ability to produce some enzymes.
Key words: medicinal plants, fungal isolates, enzymatic activity, gamma irradiation.

INTRODUCTION

Despite the great advances observed in modern synthesis-based pharmacy, the use of medicinal plants is continually expanding worldwide. The increasing search for therapeutic agents derived from plant species is justified by the emergence of diseases, yet without proper treatment, and the growth of scientific knowledge about the herbal medicines as important treatment alternatives **(Abba *et al*., 2009)**. The World Health Organization estimates that about 65-80% of the world' s population living in developing countries depends essentially on medicinal plants [herbs] for primary health care **(Kunle *et al*., 2012).**
Medicinal plants normally carry high bioburden as a result of a series of influences. By their origin, from soil, air and water may be present potentially pathogenic microorganisms to man. Microbial contamination of medicinal plants can be influenced by environmental factors such as temperature, humidity and extent of rainfall during pre-harvesting and post-harvesting periods, handling practices and the storage conditions of crude and processed medicinal-plant materials. In order to improve the purity and safety of the products, observation of basic hygiene during preparation, standardization of some physical characteristics such as moisture content, pH and microbiological contamination levels are desirable **(Kneifel *et al*., 2002; Bugno *et al*., 2006 and Abba *et al*., 2009).**The presence of microbial contaminant in non-sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the products and has the potential to adversely affect patients taking the medicines. As herbal medicinal products are complex mixtures which originate from biological sources, great efforts are necessary to guarantee a constant and adequate quality **(Busse, 2000).**
Fungi are ubiquitous and found in most foods with low and intermediate moisture contents. Fungal extracellular enzymes may play a role in biodeterioration of dried materials of medicinal plants. Protease, lipase and cellulase are considered among the most important enzymes in the degradation of organic compounds and complex materials of medicinal plants. Fungi may also produce toxic metabolites (mycotoxins), the best known are aflatoxins which are human carcinogenic **(Mankevicience *et al.*, 2007)**. Fungi also produce a variety of volatiles including alcohols, aldehydes and ketones which are often evident as moldy odors and which produce symptoms

such as headache, eye, nose and throat irritation **(Flannigan *et al*, 1991).**
From a human health stand point of view, it is recommended that, the conditions allowing fungal proliferation should be addressed and the existing fungal growth should be removed or treated. Gamma irradiation is now getting recognition throughout the world as a phytosanitary treatment of herbal materials. It improves the hygienic quality of various herbal materials and reduces the loss due to microbial contamination. Besides, it is a fast, safe, convenient and eco-friendly method. Therefore, the present work is aimed to study mycoflora of some medicinal plants and investigate the effectiveness of gamma irradiation on their enzymatic activities.

Materials and Methods

Source of medicinal plants

The medicinal plants used in this investigation, galangal, yerba mate, anise, fennel, black cumin, thymus , sinapis, feverfew, fenugreek and chamomile were purchased from the local market in Great Cairo and packed in polyethylene bags. Informations about the tested medicinal plants were presented in table (1).

Table (1) the tested medicinal plants

Common name	Scientific name	family	Traditional use	Part used
Galangal	*Alpinia glanga*	*Zingiberaceae*	As a spice, for treatment of stomach problems	Rhizomes [roots]
Yerba mate	*Ilex paraguariensis*	*Aquifoliaceae*	For lipid lowering, cancer of larynx, esophagus and oral	Leaves
Anise	*Primpinella anisum*	*Apiaceae*	As a spice, to facilitate digestion ,treatment of cold, asthma and epilepsy	Flowers
Fennel	*Foeniculum vulgare*	*Apiaceae*	For flatulence, laxative, nausea, diuretic, helps to dissolve kidney stones, antiseptic and antispasmodic	Flowers commonly known as seeds
Black cumin	*Nigella sativa*	*Ranunculaceae*	For headache , asthma, respiratory depression, expelling the urinary calculus and diuretic	Seeds
Thymus	*Thymus vulgaris*	*Labiatae*	As a gargle for laryngitis, tonsillitis and sore throats in general. For chest congestion	Leaves
Sinapis	*Brassica nigra*	*Brassicaceae*	As appetizer, diuretic, cough suppressant and for treatment of respiratory infections	Seeds
Feverfew	*Chryanthemum parthenium*	*Astraceae*	Migraine and other chronic headache	Fresh leaves and flowers
Fenugreek	*Trigonella foenum-graecum*	*Papilionaceae*	Increasing milk-flow, smothes irritated tissues, lowers fever, reduces blood sugar and has diuretic effects.	Seeds
Chamomile	*Matricaria chamomilla*	*Astraceae*	As antiinfilamatory, gentle sleep aid and for treatment of fever	Flower heads

Chemicals

Media components were purchased from Sigma Chemical Co., St. Louis, MO, USA used. All other chemicals were of reagent grade and purchased from local suppliers.

Isolation, purification and identification of fungi contaminated the tested medicinal plants

The microbiological methods were used according to Protocol for testing Ayurvedic, Siddha and Unani medicines' **(Anonymous, 2007).** Using pour plate method, samples (l0 g) were decimal diluted serially with sterile saline solution and added to Sabouraud chloramphenicol agar. Plates were inoculated and incubated at 25^{o}C for 5 days. The developing molds were isolated and counted. The fungal isolated molds were identified according to the **Manual of Clinical Microbiology (2001).**

Detection of fungal isolates enzyme activity. The enzyme activities of isolated fungi were tested using standard techniques as follow:

Protease activity

Protease activity was measured following the modified method of (**Thangam and Rajkumar, 2000).** Briefly, 1 ml cell free filtrate was added to equal amount of 1% casein in 0.2 M sodium phosphate buffer pH 7.2 and incubated at 37^{o}C for 30 min. The reaction was stopped by adding 2ml of 10% trichloroacetic acid (TCA). The resulting precipitate was allowed to settle for at least 1h. in crushed ice **(Gallop *et al*.,1957)** and the mixture was centrifuged at 4,000 rpm for 10 min. The solubilized protein in the supernatant was measured using the method of **(lowry *et al*., 1951)** at 750nm. One unit (U) of enzyme activity was defined as the amount of protease which catalyses the release of 1µg of L-tyrosine per min under the above assay conditions.

Cellulase activity

Cellulase activity was assayed by the determination of reducing sugar released from carboxymethyl cellulose (CMC). 0.5 ml of culture supernatant fluid was incubated with 0.5ml 1% CMC in 0.05 M sodium acetate buffer, pH 4.8 at 40^{o}C for 1h. The reducing sugar product was assayed by the dinitrosalicylic acid (DNSA) method **(Miller, 1959),** using glucose as the sugar standard. Controls for carbohydrate produced from substrate and of enzyme preparation were included. One unit of cellulase was defined as the amount of enzyme which produced 1 µmol equivalent per min. under the assay conditions

Lipase activity

The lipase activity was determined using the method described by **(Tietz and Fiereck, 1966).** This method was based on titrimetric estimation of free fatty acids liberated from an emulsified substrate at pH 7.4 and 37^{o}C by titration with standard alkali. One unit of enzyme activity was expressed as micro equivalents of alkali consumed per mg of protein per h of incubation.

Determination of sublethal dose of the tested fungal isolates

The irradiation process was achieved by using Co^{60} gamma source (Russian facility, Model Issledovatel) located at the National centre for Radiation Research and Technology, Nasr city, Cairo, Egypt. The dose rate of this source was (2.702 kG/h) at the time of experiment. The irradiation process was conducted as follow, under aseptic conditions; the most producer fungal isolates were cultured on Sabouraud dextrose agar [SDA] plates, and incubated at 28^{o}C for 7 days. The fungal spores were collected by flooding the agar surface with sterile saline solution, followed by gentile scraping with a sterile scraper. The resulting spore suspensions were collected by aspiration. The suspension was filtered through sterile absorbent cotton to remove mycelia fragments and spore clumps **(Cuenca Estrella *et al.*, 1999).**

Equal volumes of the recovered spore suspension (10^{5}-10^{6} spores/ ml) in duplicates were exposed to different dose levels of gamma radiation (1- 6 kGy) to determine the sublethal dose.

Effect of sublethal dose of gamma radiation on enzyme activities

The effect of γ - radiation on enzyme activities of the most active enzyme producer isolates was studied. The spore suspension of fungal isolate under study was prepared and exposed to its sublethal dose of γ- radiation and then enzyme production was conducted as mentioned before.

Stability of enzymes produced by irradiated fungal isolates

To check the stability of changes induced by γ-radiation, spore suspensions of the fungal isolates under study (which have been exposed to their sublethal doses of γ- radiation) were stored for 6 months and their enzymatic activities were determined in 2 months interval as mentioned before. Then the enzyme activities were compared with that irradiated sample without storage which represents 100 % of enzyme activity.

Statistical analysis

Data of enzyme activities were analyzed by unpaired two-tailed student's t-test **(Sendecor and Cochran, 1980).** The difference between means was considered to be statistically significant at ($p< 0.05$). Also, analysis of variance (F. test) was done for samples in each storage period using the General Linear Model **(SAS, 2002),** Least Significant Difference (LSD) and Probability ($p < \text{or} > 0.05$) were performed on the examined organisms.

Results & Discussion

Dried medicinal plants are widely used for prophylaxis or treatment of many diseases. However, the problem of dried medicinal plants contamination with microscopic fungi is still poorly understood. This contamination may result in: (1) biodeterioration of plant material; (2) production of secondary metabolites and (3) propagation of isolates with potential pathogenic properties to human and animals **(Janda *et al*., 2009).**

In the present study, a total of 17 fungal contaminants were isolated from different nine medicinal plants obtained from the market, while one medicinal plant (chamomile) was found to be free from any fungal contamination. The genera recovered were *Aspergillus, Alternaria*, *Cladosporium* and *Penicillium* and the most frequent genera were penicilli and aspergilli each recovering 50% of tested samples (table 2). These findings come in agreement with those reported by several investigators **(Dubey *et al*., 2000 and El-Zayat, 2002).**

Table (2) Isolation and Identification of fungal contaminants in the tested medicinal plants

Common name	No. of isolated fungi	Isolated fungi
Galangal	1 2	*Asp. fumigatus* *Alternaria sp.*
Yerba mate	3	*Asp. flavus*
Anise	4 5 6	*Asp. niger* *Cladosporium sp.* *P. italicum*
Fennel	7 8	*P. italicum* *P. rubrum*
Black cumin	9 10	*P. roquefortii* *P. italicum*
Thymus	11	*P. roquefortii*
Feverfew	12 13 14	*Asp. niger* *Asp. flavus* *Asp. parasiticus*
Fenugreek	15	*P. chrysogenum*
Sinapis	16 17	*Cladosporium sp.* *Asp. flavus*
Chamomile	-	-

Fungi can contaminate foods from cultivation to harvest, during transportation and storage, and in various production phases, whenever the fungus is under favorable conditions of temperature and humidity. The contamination of the materials, taken directly from nature, depends on the available surface, so that flowers and leaves contain about 100 times more contamination than fruits and seeds. The effects of fungal invasion include a reduced germination potential, development of visible moldiness, discoloration, unpleasant odor, loss of dry matter, heating, chemical and nutritional changes, loss of quality, and production of mycotoxins. In storage conditions, *Aspergillus* and *Penicillium* are predominant and the *Fusarium spp*. is an important plant pathogen {**(Frisvad & Samson (1991) and Aquino (2011)}.**

Fungal enzymes serve as invasive agents which enable the pathogens to penetrate the tissues of their hosts causing its deterioration also, it can offer information about the release of mycotoxins, depending on their physiology status, multiplication conditions and environmental factors. Screening for the production of protease, cellulase and lipase by fungal isolates as they considered among the most important enzymes in the degradation of organic compounds and complex materials of medicinal plants and the data were represented in (Table 3). The results revealed that, all isolates were able to produce these extracellular enzymes in a varying degree. It was found that species of a single genus differ in the production of enzymes. Thus, *Asp. niger* and *Asp. flavus* showed good proteolytic activity, while *Asp. fumigatus* showed weak activity.

Table (3) Detection of enzyme activities of fungal strains isolated from the tested medicinal plants.

Medicinal plant	Fungal isolate	Protease U/ml	Cellulase g/ml	Lipase U/ml
Galangal	*Asp. fumigatus* *Alternaria sp.*	3.2 4.8	0.49 0.25	75 20
Yerba mate	*Asp. flavus*	4.5	0.47	55
Anise	*Asp. niger* *Cladosporium sp.* *P. italicum*	5.6 2.3 2.3	0.45 0.22 0.43	25 35 55
Fennel	*P. italicum* *P. rubrum*	1.9 3.9	0.35 0.95	55 5
Black cumin	*P.roquefortii* *P. italicum*	3.1 2.8	1.2 0.33	65 70
Thymus	*P.roquefortii*	2.9	0.83	50
Feverfew	*Asp. niger* *Asp. flavus* *Asp. parasiticus*	5.2 4.1 4.0	0.50 0.51 1.20	30 53 5
Fenugreek	*P.chrysogenum*	2.3	0.39	35
Sinapis	*Cladosporium sp.* *Asp. flavus*	2.5 5.9	0.21 0.67	30 60

The results also showed that, *Asp. niger* and *Asp. flavus* were actively produce protease (5.6 and 5.9 U/ml), respectively and failed to do so with cellulase or lipase enzymes. However, the more efficient cellulase

producing isolates were *P. roquefortii* and *Asp. parasiticus* and (1.2 g/ml for each). It is also noted that *Asp. fumigatus* and *P. italicum* were the more efficient lipase- producing isolates (75 and 70 U/ml), respectively which showed weak activity of protease and/ or cellulase production . Our findings of high protease in *Asp. niger* agree with that by **(El-Zayat, 2002).** In general, the dried medicinal plants differed in the qualitative and quantitative composition of fungi contaminants, subsequently; fungi contaminating these materials differed in extracellular enzymes profiles.

Several thousand tons of medicinal herbs are produced annually in Egypt. These products should be of high quality and microbial purity. Use of ionizing radiation as physical method of microbiological decontamination of spices and herbs was approved by the Codex Alimentarius Commission – CDC. For fungal contamination, an average dose 5.5 kGy was enough to reduce the counts to acceptable levels (**Soriani *et al.*, 2005**). Therefore the highly producing enzyme isolates were exposed to an increasing dose of gamma radiation (1- 6 kGy) to study its effect on their survivability and to determine the subleathal dose. The results indicated that, survivability of all fungal isolates was inversely proportional to the irradiation dose (Fig. 1). Dose level 6.0 and 4.0 kGy completely destroyed the spores of *Asp. niger* and *Asp. flavus*, respectively which highly produce protease, while the spores of *P. roquefortii* and *Asp. parasiticus which* highly produce cellulose were destroyed at 6.0 and 4.0 kGy. On the other hand, the spores of *Asp. fumigatus* and *P. italicum* were destroyed at 4.0 and 6.0 kGy, respectively.

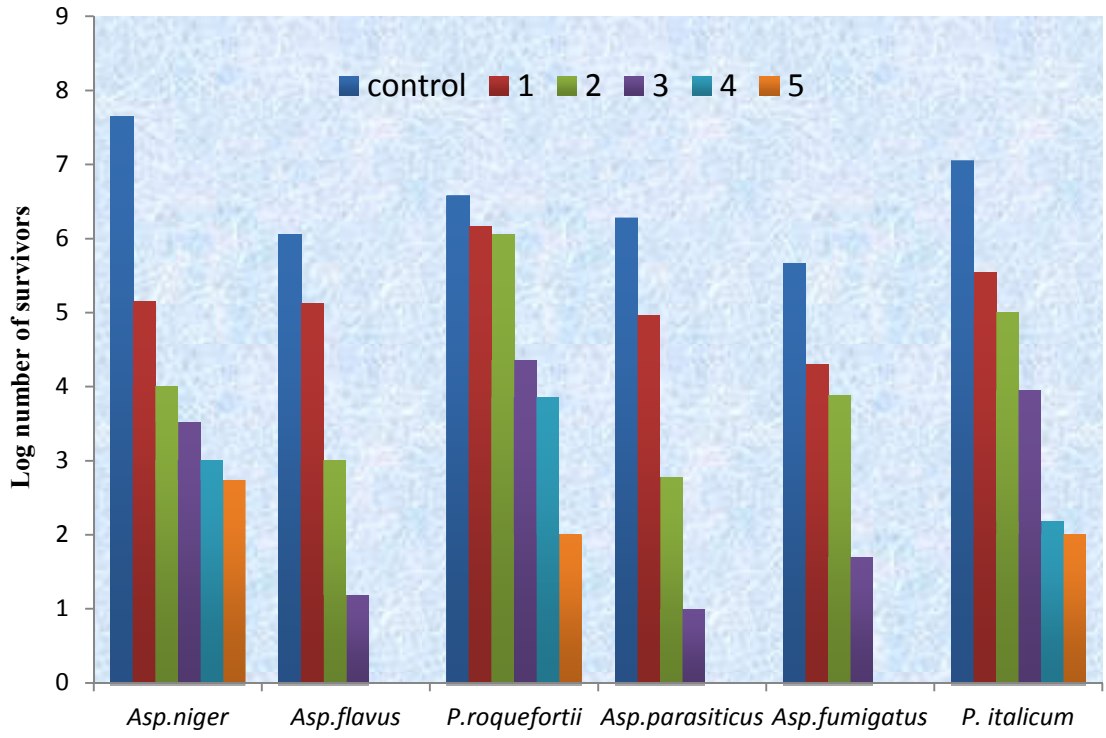

Fig. 1 effect of gamma radiation on the survivability of the highly producing enzyme isolates

Ionizing radiation reduced the viable count of bacteria and fungi. As dose increased the viable count decreased gradually **(Abo -State, 2004 and Simone *et al.*, 2005)**, these results also confirmed by **Aziz and Mahrous (2004),** they recorded that the required dose for complete inhibition of fungi ranged from 4.0 to 6.0 kGy. Similar results were documented by (**Abo -State *et al.*, 2010),** who found that, gamma radiation reduced the viable count of the spores of *Aspergillus* MAM-F23 and 35. As gamma dose increased, the viable count decreased. 5.0 and 4.0 kGy reduced the viability of the two isolates.

The data shown by **Rustom (1997)** that reported the reduction of fungal growth increased with increasing irradiation doses, but even using a dose of only 3 kGy resulted in a more than 99.9% reduction in the numbers of colony forming units of *A. flavus*. **Simone *et al.* (2005)** concluded that, Samples of corn grains were irradiated using a cobalt ^{60} source emitting gamma rays at doses of 2, 5 and 10 kGy. Gamma irradiation effectively reduces the number of colony forming units in *Asp. flavus* at all doses, but the effect was more dominant at higher irradiation doses. The lethal dose of radiation can vary according to the organism. The effectiveness of the treatment is dependent on several factors including the composition of the food, the number and type of

microorganisms and the dose applied **(Diehl *et al.*, 1994 and Aquino, 2011).**
The highly producing enzyme isolates were exposed to their sublethal doses of γ- radiation to study its effectiveness on their enzymatic activities. Table (4) recorded that, after irradiation, high significance decrease in the level of enzyme activity was recorded with all of tested strains. Our results were agreed with **Wang *et al.* (2006)** who reported that, γ- radiation inactivate some enzymes such as lipoxgenase, polyphenoloxidase and peroxidase, while there were three enzymes still active until 5 kGy. Also, **Varalakshmi *et al.* (2009)** observed that, the enzyme production by *Asp. niger* JGI 24 showed increased time of UV exposure resulted in decrease alpha amylase production. On 20 min. exposure to UV, enzyme activity was found to be zero. Low doses of gamma- irradiation may stimulate the microbial metabolic activities. Meanwhile, high doses were proved to be inhibitory for both growth and enzymatic activities of microorganisms (**El-Batal and Abdel- Karem 2001 and El- Batal and Khalaf 2003).**

Table (4) enzymatic activities of un- irradiated and irradiated fungal isolates at its sublethal doses of gamma irradiation

Fungal isolates	Tested enzyme	Enzyme activity		T- test	P- value
		Control	irradiated		
Asp. niger	Protease	9.14	5.08[b]	- 31.39	P= <0.0001
Asp. flavus	Protease	6.52	2.41[a]	- 40.87	P= <0.0001
P. roquefortii	Cellulose	0.66	0.35[b]	- 10.97	P= <0.0001
Asp. parasiticus	Cellulose	0.50	0.30[a]	- 30.21	P= 0.0016
Asp.fumigatus	Lipase	52.0	31.0[a]	-22.46	P= 0.0002
P. italicum	Lipase	68.0	36.0[b]	- 17.17	P= 0.0004

(a) Irradiation applied at 3.0 kGy (b) Irradiation applied at 5.0 kGy

Also, this study concerned with the stability of enzyme reduction induced by γ-radiation for the tested isolates. This was proved through the determination of their enzyme activities and comparing it with that irradiated sample without storage which represent 100 % and then calculated the percentage enzyme reduction in activities for each isolate. The data showed that, percentages of enzyme activities reduction of irradiated isolates at zero time storage were 44.37, 63.03, 45.13, 40.97, 40.07 and 47.22 for *Asp. niger, Asp. flavus, Asp. parasiticus, P. roquefortii, Asp. fumigatus* and *P. italicum,* respectively. It was noticed that by increasing storage time, the percentage of enzyme reduction for tested isolates was decreased with different values. Statistical analysis showed a highly significant decrease at the end of storage time (6 months), where the percentage of reduction were 2.61, 16.62, 26.32, 31.76, 9.12 and 22.14 for the previously mentioned isolates respectively. In general, it was noticed that the tested isolates exposed to 3.0 kGy can recover the changes induced in enzyme activities more than those which exposed to 5.0 kGy. The data were represented in fig.2.

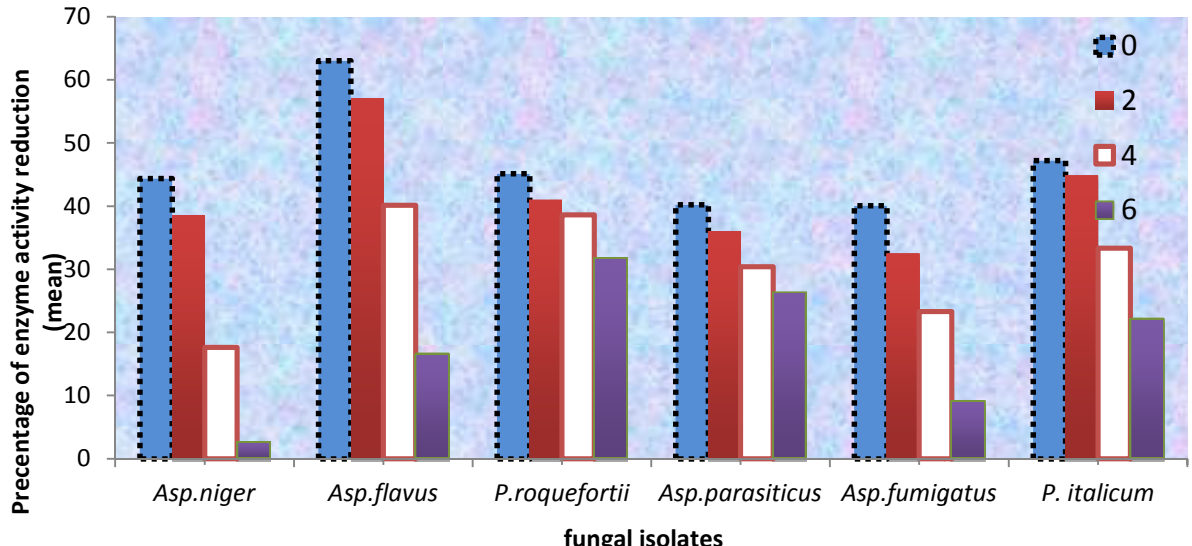

Fig.2 Stability of the enzymatic reduction by gamma radiation during storage periods/month

0 time means irradiated sample without storage
2, 4, 6 means storage time/month

In conclusion, Gamma radiation could potentially reduce the risk of fungal contamination of medicinal plants and subsequently its enzyme production. Therefore, in the insert of the human welfare and for improvement in

the quality of herbal medicines, gamma radiation at dose of 6.0 kGy is very effective treatment prior to manufacture of medicine.

Acknowledgment
The authors would like to express their appreciation to Dr. Azza, A. Shaheen, Professor of Microbiology, Radiation Microbiology Department, and National Centre for Radiation Research and Technology, Egypt, for her extensive help in the identification of fungal strains isolated in this study.

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